Inhibition of Influenza Virus Replication in MDCK Cells by Circular Dumbbell RNA/DNA Chimeras with Closed Alkyl Loop Structures

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Dedicated to Professor *Wolfgang Pfleiderer* on the occasion of his 75th birthday

We have demonstrated that a new type of circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON) with two closed nucleotide or alkyl loop structures (hexa-ethylene glycol) inhibits influenza virus A replication in MDCK cells. The enzymatic synthesis of circular dumbbell RNA/DNA chimeric oligonucleotides was achieved by enzymatically ligating a self-complementary phosphorylated oligonucleotide with T4-RNA ligase. The CDRDON-Al, with two closed alkyl loop structures, showed higher nuclease resistance, hybridization, and cellular uptake than the anti-S-ODN and the CDRDON, with two closed nucleotide hairpin-loop structures. The circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON-Al-PB2-as), containing an AUG initiation-codon sequence as the target of PB2, showed highly inhibitory effects on influenza A virus RNA expression. The limited toxicity of unmodified phosphodiester oligonucleotides and the sequence-specific binding to target mRNA indicate that circular dumbbell RNA/DNA chimeric phosphodiester oligonucleotides can be used with intact cells, and may prevent viral replication in culture.

Introduction. – Influenza is a very important infectious disease that often brings about lethal pneumonia, particularly in an aged patient or a high-risk patient suffering from a chronic respiratory disorder or heart disease. The influenza viruses are classified into three types, A, B, and C, on the basis of the differences in the serotypes of the nucleoproteins (NP) and the membrane proteins (M). Of these types, the influenza A and B viruses are prevalent every year. Influenza virus is a negative-strand RNA virus with a segmented genome. Essentially all of the transcription and replication studies of influenza virus have been carried out with A-strain viruses, which contain eight virion RNA (vRNA) segments. Viral mRNA synthesis is catalyzed by viral nucleocapsids [1], which consist of the individual vRNAs associated with four viral proteins, the nucleocapsid (NP) protein, and the three P (PB1, PB2, and PA) proteins [2]. The P proteins are responsible for viral mRNA synthesis, and some of their roles have been determined by analyses of *in vitro* reactions catalyzed by virion nucleocapsids [2][3]. The three P proteins form a complex that starts at the 3′-ends of the vRNA templates and moves down the templates in association with the elongating mRNA during transcription. The PB2 protein in this complex recognizes and binds to the cap of the primer RNA [2][3]. The PB1 protein, which was initially found at the first residue (a G residue) added onto the primer, moves as part of the P-protein complex on the 3′-ends

of the growing viral mRNA chains, suggesting that it catalyzes nucleotide addition. In addition, the P-protein complex has endonuclease activity and may function in viral mRNA synthesis [3]. On the other hand, the NP protein is the viral protein required for antitermination [4]. Consequently, NP acts by binding to the viral RNA transcript, rather than to the P protein complex and catalyzing transcription. Viral mRNA synthesis is catalyzed by four viral proteins: the nucleocapsid (NP) protein and the three P (PB1, PB2, and PA) proteins. The viral RNA polymerase (PB1, PB2, and PA) and the nucleoprotein (NP) genes of influenza virus A are potential targets for antisense oligonucleotides.

The use of oligonucleotides to modulate gene expression was first proposed by *Stephenson* and *Zamecnik* [5]. Since then, many studies have demonstrated that antisense oligonucleotides with sequences complementary to specific mRNAs (sense oligonucleotides) can be used to decrease the expression of the proteins encoded by these mRNAs, both *in vitro* and *in vivo* [6–12]. First, the antisense oligonucleotides must be sufficiently resistant to both 3′- and 5′-exonucleases, as well as the endonucleases present in serum and tissues, to avoid rapid cleavage and degradation. The antisense oligonucleotides must also have a sufficient affinity for the targeted viral mRNA to bind with a high degree of specificity and fidelity, and must be taken up by cells and tissues in adequate quantities. The oligonucleotides must, of course, evoke a mechanism of action that leads to the destruction or inactivation of the targeted mRNA, which is their biological activity. Antisense oligonucleotides with phosphorothioate backbones exhibit several advantages over the other forms, including relatively high nuclease resistance and the capacity to induce the degradation of the target sequence by RNase H [13–19]. However, phosphorothioate oligonucleotides (S-ODN) hybridize more weakly with the complementary nucleic acids than the unmodified oligonucleotides (anti-ODN) and are eventually degraded, primarily from the 3′-end. Additionally, some of the biological activities of phosphorothioate oligonucleotides cannot be attributed to an antisense mechanism of action. Many of the biological activities of phosphorothioate oligonucleotides are non-sequence-specific.

On the other hand, DNA dumbbells are stable, short segments of double-stranded DNA with closed nucleotide loops on each end, which confer exonuclease resistance [20][21]. DNA Dumbbells have historically been used as physical models for analyzing local thermal stability in DNA, for examining hairpin, cruciform, and locally melted domains within naturally occurring DNA polymers, for studying DNA conformations as substrates for various enzymes, and for overcoming the problems of double-strand oligomer dissociation when investigating nucleic acid drug targets. In addition to their utility as physical models, DNA dumbbells have biological relevance as aptamers or decoys for trapping proteins, such as transcription factors [22–27].

In this paper, we describe the design of a new class of oligonucleotides, circular dumbbell RNA/DNA chimeric oligonucleotides (CDRDON), consisting of a sense RNA sequence and its complementary antisense DNA sequence with two closed alkyl loop structures. These oligonucleotides have increased nuclease resistance, hybridization, and cellular uptake. We have also tested the inhibition of influenza A virus RNA polymerase PB2 gene expression in MDCK cells by circular dumbbell RNA/DNA chimeric oligonucleotides with two closed nucleotide or alkyl loop structures.

Results and Discussion. – *Enzymatic Synthesis of Circular Dumbbell RNA/DNA Chimeric Oligonucleotides.* The sequences of the oligonucleotides and their corresponding dumbbell structures are shown in *Fig. 1.* The synthesis of the DNA dumbbells has been achieved by enzymatic method of ligation [20][21][28–30] and a chemical (BrCN) method of ligation [30]. However, the potential toxicity of BrCN should be taken into account. Therefore, we chose the enzymatic-ligation method for the circular dumbbell RNA/DNA chimeric oligonucleotides. For the enzymatic-ligation method, the synthesis (covalent closure or formation of dumbbell duplex) can be verified by the altered electrophoretic mobility of the product (*Fig. 2*) and the resistance to enzymes (*Fig. 3*). Further proof of ligation is demonstrated by the difference in the melting-temperature profiles between the ligated and unligated structures (*Table*). The enzymatically ligated dumbbells in *Fig. 2*,*a* and *b*, migrate faster than their unligated

sense RNA 3' *aag aaa ggu aua acu uau au* 5'

anti-ODN-PB2-as 5' TTC TTT CCA TAT TGA ATA TA 3'

anti-s-ODN-PB2-as 5' TsTsCs TsTsTs CsCsAsTsAsTsTsGsAs-AsTsAsTsA 3'

NDRDON-Nu-PB2-as(a)

```
     CC TTC TTT CCA TAT TGA ATA TA CC
C                                     C
   CC aag aaa ggu a p5'    3' ua acu uau au CC
```

NDRDON-Nu-PB2-as(b)

```
     CC p5'   3' TTCTTTCCATATTGAATATA CC
C                                     C
   CC a a g  a a a  g g u  a u a  a c u  u a u  a u CC
```

NDRDON-Al-PB2-as

```
     CC TTC TTT CCA TAT TGA ATA TA CC
R                                     R
   CC aag aaa ggu a p5'    3' ua acu uau au CC
```

R= 18-O-dimethoxytritylhexaethylene glycol

CDRDON-Nu-PB2-as

```
     CC TTC TTT CCA TAT TGA ATA TA CC
C                                     C
   CC aag  aaa  ggu  aua  acu  uau  au CC
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CDRDON-Al-PB2-as

```
     CC TTC TTT CCA TAT TGA ATA TA CC
R                                     R
   CC aag  aaa  ggu  aua  acu  uau  au CC
```

Fig. 1. *The structures and the sequences of the oligonucleotides used in this study and described in the text.* Capital letters: DNA; small letters: RNA. The antisense sequences are underlined. Targeted RNA: 3′ to 5′ (*italics*); synthetic oligonucleotides: 5′ to 3′. Abbreviations: as, antisense sequence; ran, random sequence.

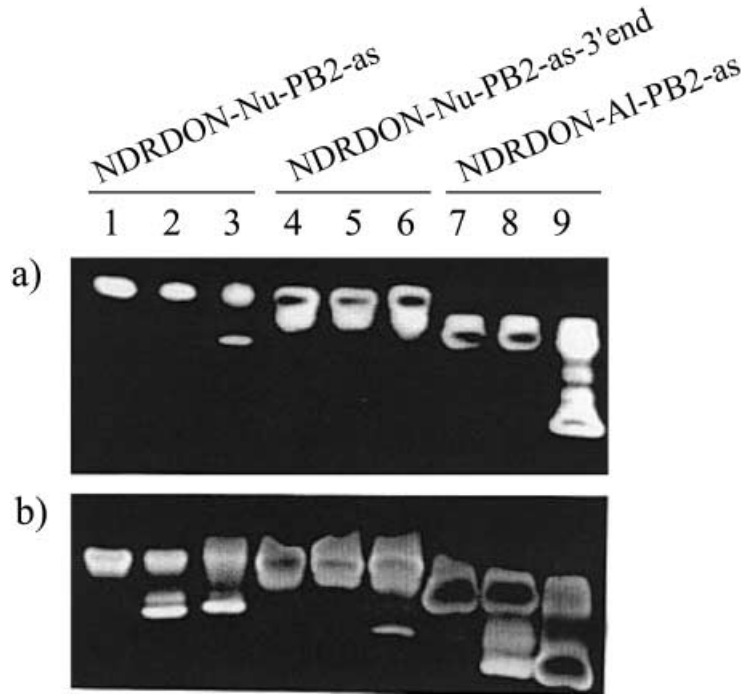

Fig. 2. *Electrophoretic analysis of the reaction mixtures after T4 ligase-induced enzymatic ligation in nicked RNA/DNA chimeric oligonucleotides (NDRDON-Nu-PB2-as* (*a* and *b*), *and NDRDON-Al-PB2-as)*. The ligation reactions were performed at room temperature for 3 h (*a*) and 24 h (*b*). Detection was performed by EtBr staining of the 10% denaturing polyacrylamide gel. In all lanes, the upper band is the unligated material and the lower band is the ligated material. *Lanes 1*, *4*, and *7* (control): nicked RNA/DNA chimeric oligonucleotides; *Lanes 2*, *5*, and *8:* nicked RNA/DNA chimeric oligonucleotides-T4 DNA ligase; *Lanes 3*, *6*, and *9:* nicked RNA/DNA chimeric oligonucleotides-T4 RNA ligase.

counterparts for all sequences shown here (upper bands, unligated materials; lower bands, ligated material). On the other hand, the ligation reaction progressed significantly in the nicked RNA/DNA chimeric oligonucleotides with the ligation site at the center (NDRDON-Nu-PB2-as), rather than at the 3′-end (NDRDON-Nu-PB2-as-3′end) of the RNA/DNA duplex. T4-RNA Ligase yield higher ligation effects than T4 DNA ligase (*Fig. 2*).

Nuclease Sensitivities of Nicked and Circular RNA/DNA Chimeric Oligonucleotides. The nuclease sensitivities of the nicked (NDRDON-Nu-PB2-as and Al-PB2-as) and circular (CDRDON-Nu-PB2-as and Al-PB2-as) RNA/DNA chimeric oligonucleotides with two closed tetra-nucleotide (Nu) and alkyl (Al, hexa-ethylene glycol) loop structures (*Fig. 1*) were studied with a 3′-exonuclease (snake venom phosphodiesterase, SVPD; *Fig. 3*). For comparison, the anti-ODN and the anti-S-ODN were chosen as controls. The anti-ODN was completely hydrolyzed within 10 min of incubation. On the other hand, the anti-S-ODN and the CDRDON-Al-PB2-as were quite stable toward exonuclease digestion even after 30 min of incubation, whereas the CDRDON-

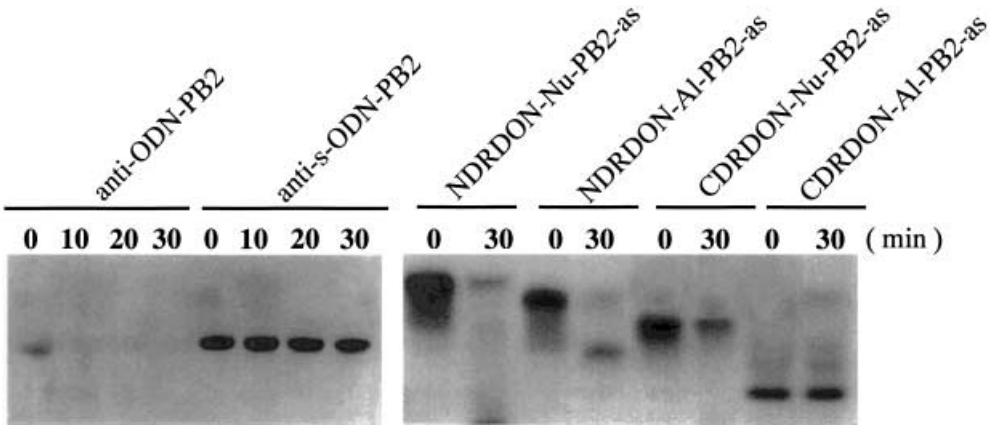

Fig. 3. *Comparison of the degradation of single-stranded (anti-ODN and S-ODN), nicked (NDRDON-Nu and Al), and circular RNA/DNA chimeric oligonucleotides (CDRDON-Nu and Al) with closed nucleotide and alkyl loop structures with 3′-exonuclease (SVPD).* These oligonucleotides were incubated with SVPD at 37° for 10–30 min. After the incubation, the oligonucleotides were loaded on a denaturing 10% polyacrylamide gel.

Table. *Melting Temperatures of Oligonucleotides*

Sequences	T_m [°][a])
anti-ODN/sens-RNA	30
anti-S-ODN/sens-RNA	26
NDRNON-Nu-PB2-as	36, 58
CDRNON-Nu-PB2-as	70
NDRDON-Al-PB2-as	46, 64
CDRNON-Al-PB2-as	74

[a]) Values were obtained in 10 mM sodium phosphate buffer and 10 mM NaCl at pH 7.0.

Nu-PB2-as showed less nuclease resistance than the CDRDON-Al-PB2-as. Furthermore, the NDRDON-Nu-PB2-as and Al-PB2-as were partially hydrolyzed after 30 min. The stability of the oligonucleotides was increased with the two hairpin loop structures with RNA/DNA base pairs (sense-RNA and antisense-DNA) in the stem at the 3′- and 5′-ends. In particular, the CDRDON-Al-PB2-as with two closed alkyl (Al, hexaethylene glycol) loop structures exhibited higher nuclease resistance than the CDRDON-Nu-PB2-as oligonucleotide with two closed tetra-nucleotide (Nu) structures.

Duplex Stabilization. The hybridization between antisense-DNA and sense-RNA in the circular dumbbell RNA/DNA chimeric oligonucleotides is the key to the nuclease resistance *in vitro* as well as *in vivo*. Furthermore, the mechanism of virus replication inhibition may involve the RNase H-mediated liberation of the antisense phosphodiester oligodeoxynucleotides from the dumbbell RNA/DNA chimeric oligonucleotides, and, therefore, the RNase H-mediated antisense phosphodiester oligonucleotides require the double stranded RNA/DNA with the dumbbell RNA/DNA chimeric oligonucleotides [31]. To assess their stability, we measured the melting temperatures of the nicked (NDRDON-Nu-PB2-as) and circular (CDRDON-Nu-PB2-as) RNA/

DNA chimeric oligonucleotides with two closed nucleotide (Nu) loop structures. The T_m values of the NDRDON-Nu-PB2-as and the CDRDON-Nu-PB2-as were 36° and 58°, and 70°, respectively. In the case of the NDRDON-Nu-PB2, two transitions were observed: one at 58°, which was typical of the hairpin stem loop between the anti-ODN and the sen-RNA with GC-rich sequences, and another at 36°, which coincided with the hairpin stem loop DNA/RNA with AT rich sequences. The CDRDON had a higher T_m value than those of the NDRDON and the DNA-RNA duplexes. On the other hand, an increase in the T_m values was also gained by exchanging the nucleotide loop with an alkyl loop. The NDRDON-Al-PB2-as and CDRDON-Al-PB2-as with closed alkyl loop structures had higher T_m values than the their counterparts with closed nucleotide loop structures. These results suggest that the stability of the oligonucleotides was increased by the introduction of the two hairpin loop structures in the RNA/DNA base pairs (sense RNA and antisense DNA) in the double-helical stem and by the exchange of the nucleotide loop for the alkyl loop.

Comparison of the Intracellular Localization of Structured Oligonucleotides. Oligonucleotides have been used as antisense inhibitors of gene expression in various culture systems and are considered to be potential therapeutic agents against cancer and infectious viral diseases. To exert any of these effects, the oligonucleotides must enter the cytoplasmic and nuclear compartments of the cells. The problem in the use of antisense oligonucleotides is that their cellular uptake is inefficient. The use of various enhancers to increase the intracellular accumulation of oligonucleotides has largely solved this problem, and has greatly facilitated the use of oligonucleotides as research tools *in vitro*. Many cellular-uptake enhancers have been reported, including cationic lipids, liposomes, peptides, dendrimers, polycations, cholesterol conjugates, and electroporation. One of the most commonly used enhancers is a mixture of a neutral lipid with a cationic lipid [32–38]. We investigated the uptake of the antisense oligonucleotides with a cationic lipid in tissue culture. The internalization efficiencies of the liposomally encapsulated FITC-labeled NDRDON-Al-PB2-as, CDRDON-Al-PB2-as, and single-stranded (anti-S-ODN) oligonucleotides were analyzed in MDCK cells. As shown in *Fig. 4*, the FITC-labeled CDRDON-Al-PB2-as strongly associated with the MDCK cells (*Fig. 4,C*), but the FITC-labeled NDRDON-Al-PB2-as did not (*Fig. 4,B*). On the other hand, the FITC-labeled anti-S-ODN also strongly associated with the MDCK cells (*Fig. 4,A*), but the fluorescent signals were weaker than those of the CDRDON-Al-PB2-as. The circularization resulting from the joining of the 3′- and the 5′-ends of the RNA/DNA chimeric oligonucleotides containing two hairpin loop structures increased the cellular uptake, as compared with the nicked RNA/DNA chimeric oligonucleotide (NDRDON-Al-PB2-as) and the single-stranded phosphorothioate (anti-S-ODN) oligonucleotide. This enhancement is significantly greater than those observed for the NDRDON and S-ODN.

Inhibition of PB2 mRNA Expression in MDCK Cells Infected with Influenza A Virus. Previously, we tested the inhibition of influenza virus RNA polymerase (PB1, PB2, PA) and nucleoprotein (NP) gene expression by circular dumbbell RNA/DNA chimeric oligonucleotides (CDRDON-Nu) with closed tetra-nucleotide loop structures, as determined by CAT protein expression (CAT activity), in the clone 76 cell line [39]. The *in vitro* activities of these oligonucleotides on the expression of the influenza A virus RNA polymerase and nucleoprotein genes were assessed on the basis of their

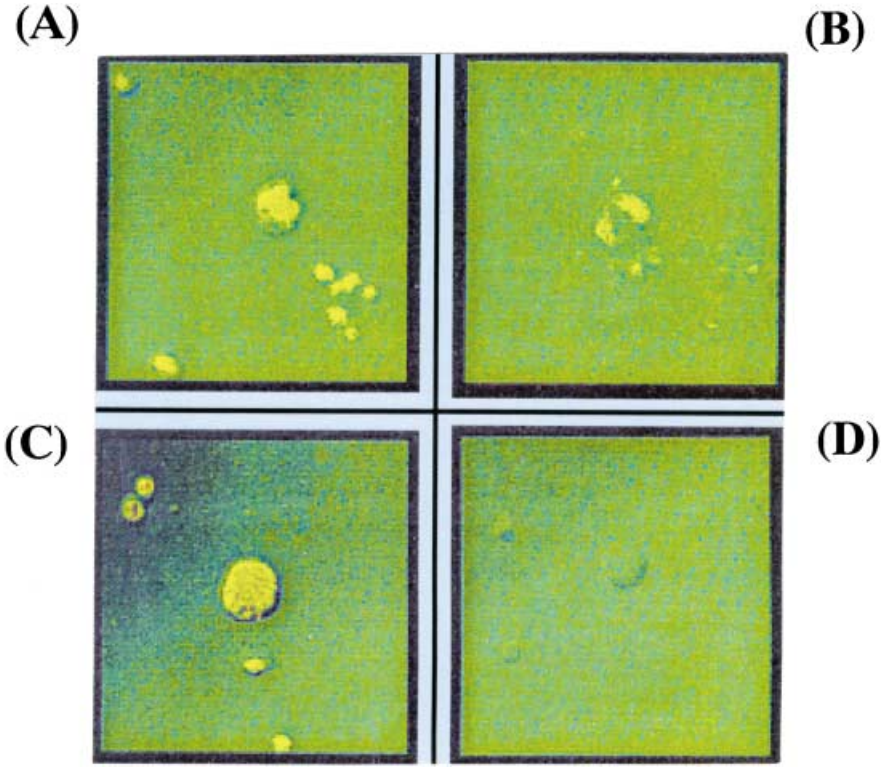

Fig. 4. *Intracellular distributions of the liposomally encapsulated single-stranded (anti-S-ODN), nicked (NDRDON-Al), and circular (CDRDON-Al) RNA/DNA chimeric oligonucleotides with two alkyl loop structures in MDCK cells.* MDCK Cells were incubated with 1 μM of liposomally encapsulated FITC-labeled-anti-S-ODN-PB2 (A), NDRDON-Al-PB2-as (B), and CDRDON-Al-PB2-as (C). MDCK cells were incubated without FITC-labeled CDRDON-Nu-PB2-as for 0 min (D).

inhibition of CAT protein expression with the CAT ELISA method. The CDRDON-Nu-PB2 was introduced into the clone-76 cells by liposome-mediated transfection. This CDRDON, containing an AUG initiation-codon sequence as the target of PB2 (CDRDON-Nu-PB2-as), yielded 60% inhibition at a 0.3 μM concentration. As control sequences, the random oligonucleotides showed no inhibitory effects on the target AUG initiation codon. A phosphorothioate oligonucleotide (anti-S-ODN-PB2), containing an AUG initiation codon sequence as the target of PB2, was chosen for comparison with the CDRDON-Nu-PB2-as. The CDRDON-Nu-PB2-as showed higher inhibitory activity than the anti-S-ODN-PB2-as (42%). These results suggest that the CDRDON conferred sequence-specific inhibition. This mechanism may involve the RNase H-mediated liberation of the antisense phosphodiester oligodeoxynucleotides from the dumbbell RNA/DNA chimeric oligonucleotides [31]. Furthermore, we observed that a pre-incubation with RNase A could involve hydrolysis of the target RNA, but does not eliminate the hydrolysis of CDRDON-gag-AUG. Consequently, this treatment does not liberate the antisense phosphodiester oligonucleotide.

In the present study, we tested the inhibitory effects of an anti-S-ODN, as well as the CDRDON-Nu-PB2-as and Al-PB2-as with closed nucleotide and alkyl loop structures, in MDCK cells infected with influenza A virus (A/PR/8/34) at an m.o.i. of 0.01, using a cationic liposome (DOTAP) delivery system [39][40]. To characterize the inhibitory effects of these oligonucleotides, we performed an Rnase-protection assay as an alternative method to quantitatively compare the mRNA levels. The results of the RNase protection assay essentially confirm the findings from the northern blot hybridization (*Fig. 5*) [39]. The PB2 mRNA was quantified by densitometric scanning. In 20 μg of total cellular RNA, the PB2 mRNA was strongly detected in MDCK cells infected with influenza A virus. The control antisense phosphodiester oligonucleotide, anti-ODN-PB2 complexed with the cationic liposome (DOTAP; *Fig. 5*,*a*, *Lane 2*), failed (18%) to inhibit the expression of the PB2 mRNA at a 10 μM concentration, whereas the treatment with 10 μM anti-S-ODN-PB2 complexed with the cationic liposome (DOTAP; *Fig. 5*,*a*, *Lane 5*) substantially reduced (80%) the amount of PB2 mRNA. However, the control sequence, the random oligonucleotide (anti-S-ODN-PB2-ran) complexed with the cationic liposome (DOTAP; *Fig. 5*,*a*, *Lane 6*), also showed inhibitory effects (54%). On the other hand, the naked anti-S-ODN-PB2-as allowed high levels of PB2 mRNA expression at a 10 μM concentration (*Fig. 5*,*a*, *Lane 7*). These results indicate that the phosphorothioate oligonucleotides exhibited inhibitory effects in a dose-dependent manner, whereas non-sequence-specific effects were conferred by treatments with high concentrations of oligonucleotides. To avoid the use of high concentrations of oligonucleotides, one approach to these problems has been the development of antisense oligonucleotides with P=O groups in the internucleotidic bonds. In addition, the degradation of phosphodiester oligonucleotides can be slowed considerably by blocking the 3′ and/or the 3′ and 5′ ends of the chain,

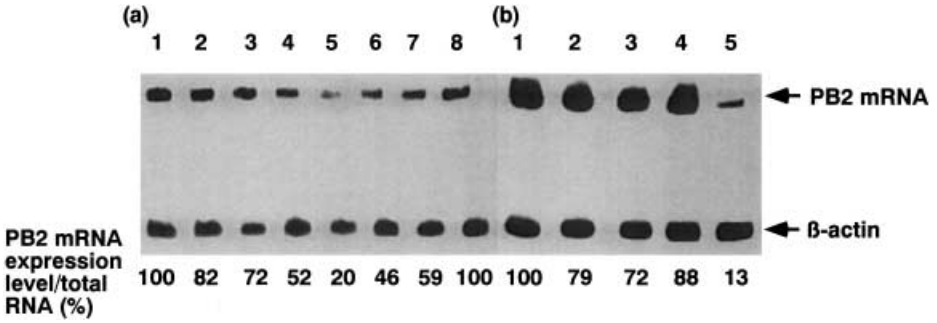

Fig. 5. *Inhibition of PB2 mRNA expression by the single-stranded (anti-ODN and S-ODN), nicked (NDRDON-Nu and Al), and circular (CDRDON-Nu and Al) chimeric oligonucleotides in influenza A virus infected MDCK cells.* The PB2 mRNA expression was measured in RNase-protection assays. Total RNA was isolated from cells that had been treated with oligonucleotides. *a*) *Lane 1* (control): cationic liposome (DOTAP) alone; *Lane 2:* 10 μM anti-ODN-PB2 encapsulated with DOTAP; *Lanes 3–5:* 3 μM, 6 μM, and 10 μM anti-S-ODN-PB2 encapsulated with DOTAP; *Lane 6:* 10 μM anti-S-ODN-PB2-ran encapsulated with DOTAP; *Lane 7:* 10 μM naked anti-S-ODN-PB2; *Lane 8:* 10 μM naked S-ODN-PB2-ran. *b*) *Lane 1* (control): cationic liposome (DOTAP) alone; *Lane 2:* 3 μM NDRDON-Nu-PB2-as encapsulated with DOTAP; *Lane 3:* 3 μM NDRDON-Al-PB2-as encapsulated with DOTAP; *Lane 4:* 3 μM CDRDON-Nu-PB2-as encapsulated with DOTAP; *Lane 5:* 3 μM CDRDON-Al-PB2-as encapsulated with DOTAP.

because the primary degradation enzymes present in cells are of the 3′-exonuclease type [41][42].

Next, we examined the effect of PB2 mRNA down-regulation by the encapsulated within nicked and circular dumbbell RNA/DNA chimeric oligonucleotides with cationic liposomes in MDCK cells infected with influenza A virus (A/PR/8/34) at an m.o.i. of 0.01 (*Fig. 5,b*). With a 3 μM concentration of the circular RNA/DNA chimeric oligonucleotide containing two alkyl loop structures (CDRDON-Al-PB2-as) encapsulated within the cationic liposome, the PB2 mRNA expression was reduced to a level similar to that of the anti-S-ODN-PB2-as, at the higher concentration of 10 μM (*Fig. 5,b*, *Lane 5*). In contrast, the nicked RNA/DNA chimeric oligonucleotides, NDRDON-Nu-PB2-as and NDRDON-Al-PB2-as (*Fig. 5,b*, *Lanes 2* and *3*), and the circular RNA/DNA chimeric oligonucleotide, CDRDON-Nu-PB2-as (*Fig. 5,b*, *Lane 4*), showed slightly inhibitory effects at a 3 μM concentration. Our previous study in clone 76 cells, with a circular dumbbell RNA/DNA chimeric oligonucleotide (CDRDON-Nu-PB2-as), showed higher inhibitory effects of RNA polymerase gene expression [31]. However, in the cases of the influenza-virus-A-infected MDCK cells, CDRDON-Nu-PB2-as inhibited influenza virus replication at a low level on the target AUG initiation codon sites. These results suggest that the tetra-C-loop (single strands) of the circular dumbbell RNA/DNA chimeric oligonucleotides (CDRDON-Nu-PB2-as) was degraded by endonucleases present in tissues [30]. In our experiment, the CDRDON-Al-PB2-as inhibited the expression of PB2 mRNA more effectively than the anti-S-ODN-PB2-as, and the nicked and circular RNA/DNA chimeric oligonucleotides with the nucleotide hairpin groups. When we performed a nuclease-protection assay as an alternative method to quantitatively compare the RNA levels, the CDRDON-Al-PB2-as substantially reduced the amount of PB2 mRNA. These results also support the sequence-specific binding to the target PB2 mRNA without the inhibition of the reverse transcriptase and/or the viral-entry process, such as, observed with the antisense phosphorothioate oligonucleotides (anti-S-ODN).

The circular dumbbell RNA/DNA chimeric oligonucleotides (CDRDON) with closed nucleotide and alkyl loop structures showed increased nuclease resistance and cellular uptake, as compared with the nicked RNA/DNA chimeric oligonucleotides (NDRDON) with closed nucleotide and alkyl loop structures, and the phosphodiester (anti-ODN) and phosphorothioate (anti-S-ODN) oligonucleotides. Of particular interest, the antisense phosphodiester oligodeoxynucleotide is liberated together with the RNA-cleavage product by RNase H treatment of the circular dumbbell RNA/DNA chimeric oligonucleotides. The liberated antisense phosphodiester oligodeoxynucleotide was bound to the target mRNA, which gave the mRNA cleavage products upon treatment with RNase H. In fact, the circular dumbbell RNA/DNA chimeric oligonucleotides exhibited higher inhibitory effects than the phosphorothioate oligonucleotides in the MDCK cells infected with influenza A virus, and showed that the nuclease resistance, the duplex stabilization, and the anti-viral activities were enhanced by changing the loop structure from a nucleotide loop to an alkyl loop. In other words, when the circular dumbbell RNA/DNA chimeric oligonucleotide is directly delivered into animal cells or virus-infected cells, its antisense phosphodiester oligodeoxynucleotide function appears. The limited toxicity of unmodified phosphodiester oligonucleotides and the sequence-specific binding to the target mRNA indicate

that circular dumbbell RNA/DNA chimeric phosphodiester oligonucleotides can be used with intact cells, and may prevent viral replication in culture.

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Experimental Part

Oligonucleotide Synthesis. The oligonucleotides were synthesized by means of the phosphoramidite approach with an *Applied Biosystems* DNA synthesizer, model *392*. Synthesis of DNA dumbbells was achieved by enzymatically ligating a self-complementary phosphorylated oligonucleotide with T4 DNA ligase [20][21][28–30]. Another method of ligation stems from the chemical template-directed cyclization with cyanogen bromide, as described by *Shaohui* and *Kool* [30].

An intramolecularly annealing (*i.e.*, self-complementary) oligonucleotide was phosphorylated (with [3-(4,4′-dimethoxytrityloxy)-2-(dicarboxyethyl)propyl] (2-cyanoethyl) (*N*,*N*-diisopropyl)phosphoramidite) and then was ligated (with T4 DNA ligase or T4 RNA ligase) to form circular oligonucleotides, as described elsewhere. The 5′-phosphorylated nicked RNA/DNA chimeric oligonucleotide (NDRDON) (0.2 A_{260}) was incubated in 100 µl of 50 mM *Tris*·HCl, pH 7.5, and 10 mM $MgCl_2$ at 90° for 10 min. The soln. was incubated with 1 µl of 100 mM ATP, and 1 µl of 25 µg BSA per ml, and 400 units of T4 DNA or T4-RNA ligase at 37° for 3 h or 24 h. The reaction mixture was extracted with an equal volume of H_2O-saturated $CHCl_3$/PhOH 1:1 (*v*/*v*), and was precipitated with EtOH. The circular oligonucleotides were purified on 10% denaturing (7M urea) polyacrylamide gels (*Fig. 2*). The ligation reaction mixtures were visualized by UV shadowing, and the products (circular) were isolated by slicing and removing the appropriate bands. The band corresponding to the desired oligonucleotide was eluted from the gel with H_2O, and was precipitated with EtOH. The identity of each ligated circular product was verified by phosphodiesterase protection mapping and measurement of the melting temperature (T_m). The synthesis of CDRDON-containing alkyl loop chains was achieved using an 18-*O*-dimethoxytritylhexaethyleneglycol-1-[2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite] as the alkylation agent.

Nuclease Stability of Oligonucleotides. The oligonucleotide (0.2 A_{260}) was dissolved in 500 µl buffer (10 mM *Tris*·HCl, pH 8.5, 10 mM $MgCl_2$, and 100 mM NaCl) and was incubated with snake venom phosphodiesterase (SVPD) at 37°. Aliquots were taken at 0, 10, 20, and 30 min and were analyzed by PAGE (20% polyacrylamide containing 7M urea). Densitometric analysis of gels stained with $AgNO_3$ was performed on a *LAS-1000* densitometry apparatus (*Fuji Photo Film Co., Ltd.*, Japan).

Cellular Uptake of FITC-Labeled Oligonucleotides. The nicked (NDRDON-Nu-PB2-as) and circular (CDRDON-Nu-PB2-as) oligonucleotides were labeled with fluorescein (Label IT™ fluorescein nucleic acid labeling kit, *Mirus*). The FITC-labeled oligonucleotides were purified by removal of the unreacted label oligonucleotides with *G50 Microspin Purification Columns*, provided with the kit. MDCK cells were diluted to 3×10^5 cells/ml in MEM containing 10% heat-inactivated FCS, and were dispensed into 6 multi-well plates. The FITC-labeled-nicked and circular oligonucleotides (1 µM) were mixed with 20 µg of DOTAP (*Roche*) in MEM and were incubated with the cells at 37° for 12 h. The cells were washed three times with PBS, resuspended in PBS containing 0.5% HCHO, and observed with a scanning confocal microscope (*MultiProbe 2001*, *Molecular Dynamics*).

Melting-Temp. Determination. Thermal transitions were recorded at 260 nm with a *Shimadzu UV-220* spectrophotometer. The inserted cell was warmed from 5° to 90° at increments of 1°, with equilibration for 1 min after attaining each temp., using a temp. controller, *SPR-8* (*Shimadzu*, Japan). Samples were heated in masked 1-cm path length quartz cuvettes fitted with *Teflon* stoppers. Each thermal denaturation was performed in 10 mM Na_3PO_4 buffer (pH 7.4), containing 100 mM NaCl and the oligomer at a concentration of 0.2 A_{260}. The oligomer was kept at 90° for 5 min, and then was cooled to 5°. At temp. below 20°, N_2 gas was continuously passed through the compartment to prevent the formation of condensate. Absorbance values were normalized and plotted against temp.

Virus and Cells. Madin-Darby canine kidney (MDCK) cells were kindly provided by Dr. *K. Nerome* (National Institute of Health, Tokyo, Japan). The cells were grown as a monolayer stationary culture in *Eagle*'s MEM supplemented with 7.5% $NaHCO_3$, 10% fetal calf serum, and 20 mg/ml fungizone. Influenza A/PR/8/34 (H1N1) virus was grown in the allantoic cavity of 10-day-old embryonated chicken eggs for 48 h at 35°. The allantoic fluid was clarified at 6000 g for 15 min and was stored at −80°.

RNase Protection Assay [39]. MDCK Cells (1×10^6 ml^{-1}) were grown in a 96-well microtiter tray and were treated with MEM containing 10% FCS at 34°. After 24 h, the cells were washed with PBS, a mixture of oligonucleotides and lipofection reagent (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium-methyl sulfate; DOTAP, *Boehringer Mannheim*) in MEM (200 µl) was added, and the cells were incubated for 4 h at 34°. The cells were washed with PBS, and each well was infected with 200 µl of virus suspension at a multiplicity of infection (MOI) of 0.01. All experiments were performed in triplicate. The cells were incubated for four days at 34° in a CO_2 incubator. To produce the single-stranded ribo-probes, 71 bp DNA fragments containing the AUG initiation codon of the PB2 mRNA were synthesized by the phosphoramidite method, with an *Applied Biosystems* model *392* DNA/RNA synthesizer. The digoxigenin (DIG)-labeled riboprobe (52 bases with the AUG initiation codon) was produced by *in vitro* transcription with T7 RNA polymerase. The probe was purified by electrophoresis on a 10% denaturing polyacrylamide (7M urea) gel and was then eluted by shaking in H_2O at 37° overnight. The soln. containing the probe was extracted once with an equal volume of PhOH/$CHCl_3$/isoamyl alcohol 25 : 24 : 1 (*v*/*v*), and the probe was precipitated with EtOH and redissolved in DEPC-treated H_2O. The probe (120 pg) was mixed with 20 µg of total cellular RNA to perform the RNase-protection assay using the HybSpeed RPA kit, as described by the manufacturer (*Ambion Inc.*, Austin, TX). These assays were performed using the β-actin probe (360 bp containing 250 bp of sequence complementary to the β-actin gene) as a control. Densitometric analysis of gels was performed on a *LAS-1000* densitometry apparatus (*Fuji Photo Film Co., Ltd.*, Japan).

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